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The Preparation of a Safe and Efficient Antirabic Vaccine

BY

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CONTENTS.

The Preparation of a Safe and Efficient Antirabic Vaccine.

CHAPTER I.

Introduction.

THE two cardinal factors to be taken into consideration in the preparation and administration of vaccines whether for prophylactic or curative purposes are, *safety* and *efficiency*. When Pasteur first applied his method of antirabic treatment to man these were the two points upon which he concentrated his attention, with the result that he firmly established the principles of antirabic treatment now carried out in almost every civilised country where rabies is found.

In the practice of conferring a specific immunity on man and animals by inoculating them with dead bacterial vaccines, the question of safety, or freedom from any risk of communicating the disease which the vaccine is intended to prevent is foreclosed, and the main points to be taken into consideration are a proper system of dosage, and the interspacing of doses; but when living vaccines are used the question assumes a different aspect.

It is now well established that dead bacterial vaccines are efficient in producing an immunising response in man and animals; a response which in most cases it would not be justifiable to attempt to produce with living bacteria. On this account it is now almost the universal custom in the prophylactic and curative vaccination of infectious diseases in man to make use of killed cultures of the causal micro-organisms. No person would be justified in using a living staphylococcus, or a living streptococcus vaccine, when dead vaccines prepared from these germs answer every purpose.

In the immunisation of animals it is not an uncommon practice to begin by using dead bacterial cultures, and then go on to living cultures; but in man it is exceptional to use living microbes either for prophylactic or curative purposes, although in some cases it has been done with perfect safety, *e.g.*, in the prophylaxis of cholera and typhoid fever. In some cases an attenuated living vaccine is used as a prophylactic by communicating a modified form of the disease, *e.g.*, vaccinia to protect against small-pox in man; but in this particular case the person inoculated can afford to allow the virus to multiply without running any risks, and moreover, the multiplication of the living element in this vaccine is no doubt the strong-

est point in favour of its efficiency. In the other two examples given, *viz.*, in the prophylaxis of cholera and typhoid fever in man, in which living vaccines have been injected subcutaneously, a multiplication is the last thing to be desired, and as far as we know it does not take place when these vaccines are given subcutaneously; but even so, it is now the custom in the prophylaxis of typhoid and cholera to use only dead bacterial cultures.

When we apply these facts to antirabic treatment, the subject assumes an aspect worthy of careful consideration, because the multiplication of a living rabies virus intended as a prophylactic vaccine would mean hydrophobia and death to the person inoculated.

Pasteur recognised this danger, and provided against its occurrence by commencing treatment with a dead virus, followed by an attenuated living virus. By this means he was able to commence the immunisation of his patients with a vaccine which was perfectly safe and which established a degree of immunity sufficient to render the subsequent injection of living and virulent virus also quite safe.

Sometime after Pasteur inaugurated his system, Högyes of Buda-Pesth introduced a method of antirabic treatment by injecting high dilutions of living and virulent virus to begin with, gradually going on to lower dilutions. This method has been adopted by several Pasteur Institutes, and has been reported upon favourably by some observers. It has the merit of being easier to carry out, and is less expensive than the treatment advocated by Pasteur and his followers; but whether it is the best and safest method that science can devise in the absence of exact knowledge about the causal micro-organism of rabies, is a disputed point.

We know that rabies is due to a living virus which has never been cultivated outside the tissues of man or animals suffering from the disease; we also know that many of the symptoms present in rabies resemble those present in cases of tetanus.

In tetanus the symptoms depend upon extra-cellular toxines elaborated by the tetanus bacillus: but whether any or all of the symptoms of rabies depend upon extra-cellular toxines elaborated by the causal micro-organism in a manner somewhat similar to those of tetanus is a subject about which we know practically nothing, and we are not likely to gain much information on this point until it becomes possible to cultivate the causal micro-organism of rabies in artificial media, and to experiment with pure cultures separate from the nerve tissue of animals dead of the disease.

In the meantime it only remains to follow the lines laid down by Pasteur, but modified in accordance with the experience gained from recent advances in our knowledge of prophylactic inoculations in other diseases.

Those who advocate the "dilution method" (Högyes' method) of treatment, take it for granted that a rabies toxine separate from the living virus does not exist;

and those who advocate the "dried cord method" (Pasteur's method) keep in view the possibility of the existence of a toxine in the nerve centres (brain and spinal cord), the principal seat of the virus and where it multiplies. Moreover, there is another point which influences the selection of a method of treatment, *viz.*, the attenuation of rabies virus.

In the dried cord method of preparing a vaccine, attenuation of the virus is supposed to take place from day to day, and strictly in accordance with the length of time the cord has been dried under fixed conditions; but here, again, the occurrence of attenuation is denied by some and firmly believed in by others. It would serve no useful purpose to enumerate the arguments for and against these different views. The cultivation of the virus in artificial media (should this ever become possible) is the source from which the gaps in our knowledge about rabies virus are likely to be filled in; and until this has been accomplished the views of workers on rabies are sure to differ widely.

Setting aside these two methods of preparing an antirabic vaccine, *viz.*, the "dried cord method" and the "dilution method," in both of which a living virus is used, are there any other possible methods of preparing a safe and efficient vaccine?

As I have already stated dead vaccines have been found safe and efficient in the prophylaxis of other diseases, why should not similar methods apply to rabies?

With the object of throwing some light upon this important question the experiments upon which this paper is based were carried out. In a word, my object was to ascertain whether animals can be as highly immunised with a dead virus as with a living virus: and if this is possible it would naturally follow that such a vaccine would be safe and efficient, two most important and essential properties in an antirabic vaccine, or in any other vaccine.

We know that heat is a damaging agent to use in killing bacterial vaccines when it is necessary to store them for some time before use; and that carbolic acid in weak dilutions is a reliable agent for sterilising bacterial vaccines without lessening their immunising and keeping properties.[1] On the other hand, a low degree of heat, any temperature below 60 C, applied from 15 to 30 minutes does not materially diminish the immunising properties of a bacterial vaccine intended for early use; but after heating it is always necessary to add an antiseptic when the vaccine has to be stored, or sent out for use.

In those vaccines which are easily killed in a small percentage of an antiseptic, such as from 0·5 to 1 per cent. carbolic acid, it is unnecessary to use heat at all, because it would not do away with the necessity of afterwards adding an anti-

[1] Semple & Matson: On the Preparation and Keeping Properties of Antityphoid Vaccines.—*The Lancet*, August 14, 1909.

septic to preserve the vaccine, and to guard against any subsequent contamination. In preparing a dead antirabic vaccine it was necessary to make a choice between these two methods, *viz.*, heat followed by an antiseptic, or an antiseptic only.

In the experiments which follow it will be seen that the rabies virus is easily killed by heat, and that a temperature of 50°C for 15 minutes is sufficient to destroy it. It will also be seen that it is more resistant to carbolic acid at room temperature than most non-spore forming bacteria; but at a temperature of 37°C it is easily killed in 1 per cent. carbolic acid.

Of these two methods of killing a vaccine, *viz.*, by heat, and by carbolic acid, carbolic acid is the one which produces least change in the vaccine.[1]

When heat is employed we make use of an agent of the effect of which we know nothing whatever, except that it kills the living element, and that bacterial vaccines killed by heat retain their immunising properties for a shorter period than similar vaccines killed by carbolic acid.

For these reasons it was decided to experiment with a vaccine prepared by killing the virus in 8 per cent. dilutions in normal saline solution to which had been added 1 per cent. carbolic acid. Twenty-four hours at a temperature of 37°C was found to be sufficient to kill the virus in 8 per cent. dilutions when 1 per cent. carbolic acid had been added. After 24 hours the killed vaccine was diluted with an equal volume of sterile normal saline solution, and kept at room temperature for use when required. This gave a 4 per cent. dilution of virus in 0·5 per cent. carbolic acid normal saline solution; a dilution suitable for treatment purposes, or which could be still more diluted if considered necessary.

In a preparation of this kind the vaccine will keep for months, and retain its immunising properties unimpaired, provided that it is kept in a moderately cool place screened from light. That it is a safe and efficient vaccine in the immunisation of animals the experiments recorded in this paper afford ample proof.

It also possesses the great advantage that it could be sent from the laboratory where it is prepared to places at a distance where any medical man with a good knowledge of the administration of vaccines could carry out the treatment of patients bitten by rabid animals. When we realise what this means in a country like India, where rabies is so widely scattered and of common occurrence, the advantages of such a system in providing a safe and efficient vaccine are enormous. Instead of having a number of Pasteur Institutes scattered over the country it would be necessary to have only a Central Institute where the vaccine could be prepared and sent to other centres where treatment could be carried out. The expenses connected with these centres would be very small, long and costly

[1] **Semple & Mateon: On the Preparation and Keeping Properties of Antityphoid Vaccines.—*The Lancet*, August 14, 1909.**

journeys would be avoided, and most important of all, the patients would come under early treatment, and a treatment free from risks.

In the preparation of a dead rabies vaccine it is necessary to know what are the effects on living virus of agents such as heat and carbolic acid which could be used in killing the virus; and in testing the rabicidal properties of the serum of animals immunised with a rabies vaccine it is necessary to know what are the effects of normal saline solution (the fluid used to prepare dilutions) on the virus dilutions used for testing the serum. The experiments recorded in Tables I to IX answer these questions. The experiments recorded in Series I to V prove that animals, such as monkeys, dogs, and rabbits, can be highly immunised with a dead rabies vaccine, killed and preserved in carbolic acid.

Other workers have also obtained evidence of the immunising properties of dead rabies virus.

Poor,[1] recording his own experiments with heated virus, in a recent article (September 1910) mentions, that, "Fermi as the result of his experiments recommended the use of dead virus only; his plan being to kill the virus with carbolic acid, and store the emulsions for future use."

The same author also mentions that, "Otto Heller in his work on protective inoculation against rabies (Jena, 1906) gives the experience of others in the use of virus killed in various ways; *e.g.*, heat, and by glycerine; and his own experiments in the use of virus killed by grinding according to the method of McFadyen." Heller used the brains of rabbits after death from "fixed virus" infection.

[1] Poor—The Immunising Properties of Killed Rabies Virus. Collected Studies from the Research Laboratory, Department of Public Health, City of New York. Vol. IV. Issued September 1910.

CHAPTER II.

The Effect of Heat on Rabies Virus.

TABLE I

It has long been known that rabies virus is easily destroyed by heat. This is a fact which has been used to prove that the causal organism of rabies (whatever it may eventually turn out to be) cannot be a spore-forming organism, for we know that all spores require a high temperature before they are destroyed, and that many of them require a boiling temperature before they are even injured, and a few kinds require boiling for one or two hours before their destruction is finally accomplished.

The very fact that rabies virus is so sensitive to heat puts the causal organism out of the category of spore-forming germs. This sensitiveness to heat of a virus about the nature of which we know so little would make one hesitate before using heat as an agent in the preparation of a dead vaccine, a vaccine in which an extracellular toxine may possibly be an important factor in its immunising properties. Marie[1] states that the destruction of the virus begins at a temperature of 45°C, and that 24 hours' exposure to this temperature completes its destruction. Marie also mentions the following results of the effects of heat on rabies virus obtained by other workers: — On peut le stériliser par une chauffage de 5 minutes à 48°, de une heure à 50° (Celli) de quelques minutes à 60° (Roux), de trente minutes à 52-58° (Hogyes). It will be seen that there is a wide range of difference in the examples given; from 5 minutes at 48° C to 1 hour at 50° C (Celli) several minutes at 60° C (Roux) and 30 minutes at 52° to 58° C (Hogyes).

The results of my own experiments on this point are given in Table 1. On referring to this table it will be seen that a 5 per cent. emulsion of fixed virus from the medulla of a rabbit is destroyed when heated in a water bath at a temperature of 50° C in 15 minutes, and that a similar emulsion is not even injured in 15 minutes at a temperature of 45° C. In the preparation of 5 per cent. emulsions used in the experiments referred to in Table I one gramme was taken from the medulla of a rabbit soon after it had died from "fixed virus" rabies, it was ground up with a pestle and mortar in 20 c.c. sterile normal saline solution, and then passed through a fine wire strainer to remove any rough particles of fibrous tissue, etc. A few c.c. were then transferred to several sterile test tubes, and heated in a water bath at the temperatures, and for the times specified in the table. A few minutes after removal from the water bath, 0·2 c.c. was used to inoculate healthy full grown rabbits subdurally, with the results recorded in Table 1. A control rabbit

[1] Marie—L'Etude Experimentale de la Rage, 1909, pp. 105-106.

was also inoculated subdurally with 0·2 c.c. of the 5 per cent. virus emulsion before heating; and this rabbit and the one inoculated with 0·2 c.c. of the virus after heating at 45°C for 15 minutes both developed rabies on the seventh day. Five other rabbits inoculated subdurally with a similar amount of the same virus, but heated either at a temperature of 55°C or 50°C for 30 or 15 minutes, remained well.

The results of these experiments show that a 5 per cent. emulsion of fixed virus in normal saline solution is destroyed at a temperature of 50°C in 15 minutes, but not at a temperature of 45°C in the same time.

TABLE I.

Experiments to test the effects of heat on "fixed rabies virus" when emulsified in normal saline solution.

No. of experiment.	Percentage of "fixed virus" in normal saline solution used.	Time and temperature at which heated before being used.	Animal inoculated.	Amount inoculated, and method of inoculation.	Result.	Remarks.
	Per cent.					
1	5	55°C for 30 minutes.	Rabbit .	0·2 c.c. subdurally.	Remained well.	
2	5	55°C for 15 minutes.	Ditto.	Ditto.	Ditto.	
3	5	50°C for 30 minutes.	Ditto.	Ditto.	Ditto.	
4	5	50°C for 15 minutes.	Ditto.	Ditto.	Ditto.	
5	5	50°C for 15 minutes	Ditto.	Ditto.	Ditto.	
6	5	45°C for 15 minutes.	Ditto.	Ditto.	Rabies, 7th day.	
7 Control	5	Not heated . .	Ditto.	Ditto.	Rabies, 7th day.	

A 5 per cent. dilution of fixed rabies virus in normal saline solution is destroyed at a temperature of 50°C in 15 minutes, but not in the same time at 45°C

CHAPTER III.

The Effect of Carbolic Acid on Rabies Virus.

The fact that carbolic acid destroys rabies virus has never been doubted, but in order to kill the virus for the purpose of making a vaccine, the dilution of carbolic acid, the dilution of virus to use, the temperature to which the mixture should be exposed and the time limit of exposure require careful consideration. The less the change produced in any vaccine used for immunising purposes the better. This is a principle to be kept in mind when the object is to obtain a dead but otherwise unaltered and efficient vaccine. It is necessary that the active principle and original properties of the vaccine, once it has been rendered safe for use, should be interfered with as little as possible, and as so much depends upon the immunising properties of a rabies vaccine the utmost care should be taken in its preparation.

In order to place the subject on a sound footing, it was first of all necessary to experiment with various dilutions of rabies virus to which had been added various percentages of pure carbolic acid, the mixtures of virus and carbolic acid being kept for variable periods either at room temperature, or a temperature of 37°C. The rabies virus used was "fixed virus" taken fresh from the medulla of rabbits after death from "fixed virus" rabies.

A portion of the medulla was removed a few hours after death, weighed, then ground up in a mortar with sterile normal saline solution to which had been added the percentage of carbolic acid to be tested. The emulsions were strained through a fine wire strainer, and filled into bottles which were then capped with rubber, and finally exposed to whatever temperature it was desired to test. By this means it was easy to obtain any dilution of virus, and in any percentage of carbolic acid desirable; it was also easy to remove a sample for experimental purposes from any of the bottles by puncturing the rubber cap with the needle of a syringe and withdrawing the piston.

The test applied in all cases was the subdural inoculation of a rabbit with 0·2, or 0·25 c.c. of the carbolised virus emulsion.

Healthy full grown animals were used in all these experiments. The animals were anæsthetised, trephined and the amount specified injected under the duramater. The first symptom of paralysis was taken as the first sign of rabies, although in some cases it might have been possible to arrive at a diagnosis by taking into consideration other and earlier symptoms, such as tremors of the head, when the animals were disturbed.

TABLE II.

This table gives the results of nine experiments with 1 per cent. dilutions of virus in 0·5 per cent. carbolic acid normal saline solution when the mixture

had been kept for various periods up to 20 days at room temperature; also the result of one experiment with 0·5 per cent. rabies virus in 0·5 per cent. carbolic acid when the mixture had been kept for 24 hours at room temperature. It will be seen that under these conditions 1 per cent. rabies virus in 0·5 per cent. carbolic acid retains its virulence unimpaired up to 12 days, and is not destroyed after 20 days, although it had by that time lost somewhat in virulence, as shown by a 10 days' incubation period when a rabbit was inoculated subdurally, instead of 7 days, the normal period after which a rabbit shows symptoms of paralysis when inoculated subdurally with "fixed virus."

In experiment 9 it will be seen that 0·5 per cent. virus in 0·5 per cent. carbolic acid retains its virulence unimpaired at room temperature for 24 hours at least.

TABLE II.

Experiments to test the effect of carbolic acid on "fixed rabies virus" when kept at room temperature (about 21° C).

No. of experiment.	Percentage of "fixed virus" in normal saline solution used.	Percentage of carbolic acid added.	Time and temperature at which kept before being used.	Animal inoculated.	Amount inoculated, and method of inoculation.	Result.	Remarks.
	Per cent.	Per cent.					
1	1	0·5	24 hours, room temp., about 20°C.	Rabbit	0·2 c.c. subdurally.	Rabies, 7th day.	
2	1	0·5	48 hours, room temp., about 20°C.	Ditto.	Ditto.	Ditto.	
3	1	0·5	3 days, room temp., about 20°C.	Ditto.	Ditto.	Ditto.	
4	1	0·5	4 days, room temp., about 20°C.	Ditto.	Ditto.	Ditto.	
5	1	0·5	5 days, room temp., about 20°C.	Ditto.	Ditto.	Ditto.	
6	1	0·5	10 days, room temp., about 20°C.	Ditto.	Ditto.	Ditto.	
7	1	0·5	12 days, room temp., about 20°C.	Ditto.	Ditto.	Ditto.	
8	1	0·5	20 days, room temp., about 20°C.	Ditto.	Ditto.	Rabies, 10th day.	
9	½	0·5	24 hours, room temp., about 20°C.	Ditto.	Ditto.	Rabies, 7th day.	

A 1 per cent. dilution of "fixed rabies virus" in normal saline solution can survive for at least 20 days in 5% per cent. carbolic acid at room temperature (about 20°C).

TABLE III

In the nine experiments recorded in this table, seven were carried out with 2 per cent. dilutions of virus in 1 per cent. carbolic acid; and two with 4 per cent dilutions of virus in 2 per cent. carbolic acid, when the mixtures had been kept at room temperature for various periods.

The results show that a 2 per cent. dilution of virus retains its virulence unimpaired in 1 per cent. carbolic acid at room temperature for 3 days, but begins to lose it after 4 days and is killed in 6 days; and that a 4 per cent. dilution of virus in 2 per cent. carbolic acid is killed in 24 hours at room temperature.

From these results it is evident that the amount of carbolic acid (2 per cent.) necessary to kill a 4 per cent. dilution of rabies virus in 24 hours at room temperature is too large an amount to be retained in a vaccine intended for antirabic treatment; on this account it was decided to test the effects of a reduction in the per entage of virus, and not to increase the carbolic acid beyond 1 per cent. (see Table IV).

TABLE III

Experiments to test the effect of carbolic acid on "fixed rabies virus" when kept at room temperature (about 20° C).

No. of experiment	Per cent. of fixed virus in final dilution	Percentage of carbolic acid added	Time and temperature at which kept before being used	Animal inoculated	Amount inoculated and method of inoculation.	Result.	Remarks
	Per cent	Per cent					
1	2	1	24 hours, room temperature, about 20° C.	Rabbit	0·2 c.c., subdurally.	Rabies, 7th day	
2	2	1	48 hours, room temperature, about 20° C.	Ditto.	Ditto	Ditto.	
3	2	1	3 days, room temperature, about 20° C.	Ditto.	Ditto	Ditto.	
4	2	1	4 days, room temperature, about 20° C.	Ditto.	Ditto.	Rabies, 8th day.	
5	2	1	6 days, room temperature, about 20° C.	Ditto.	Ditto	Remained well.	
6	2	1	9 days, room temperature, about 20° C.	Ditto.	Ditto.	Ditto.	
7	2	1	12 days, room temperature, about 20° C.	Ditto.	Ditto.	Ditto.	
8	4	2	24 hours, room temperature, about 20° C.	Ditto.	Ditto.	Ditto.	
9	4	2	24 hours, room temperature, about 20° C.	Ditto.	Ditto	Ditto.	

A 2 per cent. dilution of "fixed rabies virus" in normal saline solution can survive for at least 4 days, but not for 6 days in 1 per cent. carbolic acid at room temperature (about 20°C), but a 4 per cent. is destroyed in 24 hours in 2 per cent. carbolic acid at room temperature

TABLE IV.

This presents a series of experiments carried out with 1 per cent. dilutions of rabies virus in 1 per cent. carbolic acid normal saline solution when kept at room temperature for various periods.

The results of this series show that a 1 per cent. dilution of virus retains its virulence unimpaired for 3 days in 1 per cent. carbolic acid at room temperature but begins to lose it after 4 days, and is killed in 6 days, therefore a vaccine prepared by this method would require 6 days, and it would then have to be diluted with an equal volume of normal saline solution so as to reduce the amount of carbolic acid to a workable percentage for antirabic treatment. This would give 0·5 per cent. dead rabies virus in 0·5 per cent. carbolic acid after waiting for 6 days. A vaccine prepared by this method would be too dilute for efficient treatment, so the method was discarded.

An efficient rabies vaccine should contain at least 2 per cent. rabies virus, and not more than 0·5 per cent carbolic acid when administered to patients. To obtain such a vaccine by killing the virus in carbolic acid at room temperature in 24 hours without having to add too large a percentage of carbolic acid in the first instance would be impossible. Marie[1] states that rabies virus in 1 per cent. dilutions is not destroyed in 2·5 per cent. carbolic acid in 24 hours at room temperature.

TABLE IV.

Experiments to test the effect of carbolic acid on "fixed rabies virus" when kept at room temperature (about 20° C).

No. of experiment.	Percentage of "fixed virus" in normal saline solution used	Percentage of carbolic acid added.	Time and temperature at which kept before being used.	Animal inoculated.	Amount inoculated, and method of inoculation.	Result.	Remarks.
	Per cent	Per cent					
1	1	1	24 hours, at room temperature, about 20°C.	Rabbit	0·2 c.c., subdurally.	Rabies, 7th day.	
2	1	1	48 hours, at room temperature, about 20°C.	Ditto.	Ditto .	Ditto.	
3	1	1	3 days, at room temperature, about 20°C.	Ditto.	Ditto .	Ditto.	
4	1	1	4 days, at room temperature, about 20° .	Ditto.	Ditto .	Rabies, 8th day.	
5	1	1	6 days, at room temperature, about 20°C.	Ditto.	Ditto .	Remained well.	
6	1	1	9 days, at room temperature, about 20°C.	Ditto.	Ditto .	Ditto.	
7	1	1	12 days, at room temperature, about 20°C.	Ditto.	Ditto .	Ditto.	

A 1 per cent. dilution of "fixed rabies virus" in normal saline solution can survive for at least 4 days, but not for 6 days in 1 per cent. carbolic acid at room temperature (about 20°C).

[1] Marie—L'Etude Experimentale de la Rage, 1909, page 125.

TABLE V.

Owing to the results obtained from the experiments given in Tables II, III, and IV with dilutions of rabies virus in various percentages of carbolic acid, it was necessary to try the killing effects of carbolic acid on rabies virus at a temperature of 37°C. It is a well known fact that rabies virus can survive for a prolonged period in glycerine at room temperature, but is quickly killed in glycerine at a temperature of 37°C. It will be seen from the experiments which follow that the killing power of carbolic acid on rabies virus is also increased at a temperature of 37°C. In this series of twelve experiments, four were carried out with 0·5 per cent. carbolic acid added to 2 per cent., 1 per cent., and 0·5 per cent. dilutions of rabies virus; and eight with 1 per cent. carbolic acid added to 1 per cent., 2 per cent., and 4 per cent. dilutions of rabies virus. All these carbolised emulsions of virus were kept for 24 hours at a temperature of 37°C before being used to inoculate rabbits subdurally with 0·25 c.c.

On referring to the table it will be seen that 0·5 per cent. carbolic acid does not kill rabies virus in 24 hours at a temperature of 37°C even when the virus is tested in 0·5 per cent. dilutions; and that 1 per cent. carbolic acid kills a 4 per cent. dilution of rabies virus in 24 hours at this temperature (37°C).

The result of this last experiment showed that it was reasonable to expect that 1 per cent. carbolic acid at a temperature of 37°C would probably kill stronger preparations of the virus, and as this point was of practical importance in the preparation of a stronger vaccine it was necessary to investigate still further the killing properties of carbolic acid at a temperature of 37°C.

TABLE V.

Experiments to test the effect of carbolic acid on "fixed rabies virus" when kept at a temperature of 37°C for 24 hours.

No. of experiment.	Percentage of "fixed virus" in normal saline solution used.	Percentage of carbolic acid added.	Time and temperature at which kept before being used.	Animal inoculated.	Amount inoculated, and method of inoculation.	Result.	Remarks.
	Per cent.	Per cent.					
1	2	0·5	24 hours at 37°C	Rabbit	0·25 c.c.	Rabies, 9th day.	
2	2	0·5	Ditto.	Ditto.	Ditto.	Ditto.	
3	1	0·5	Ditto.	Ditto.	Ditto.	Rabies, 7th day.	
4	½	0·5	Ditto.	Ditto.	Ditto.	Rabies, 8th day.	
5	1	1	Ditto.	Ditto.	Ditto.	Remained well.	
6	2	1	Ditto.	Ditto.	Ditto.	Ditto.	
7	4	1	Ditto.	Ditto.	Ditto.	Ditto.	
8	4	1	Ditto.	Ditto.	Ditto.	Ditto.	
9	4	1	Ditto.	Ditto.	Ditto.	Ditto.	
10	4	1	Ditto.	Ditto.	Ditto.	Ditto.	
11	4	1	Ditto.	Ditto.	Ditto.	Ditto.	
12	4	1	Ditto.	Ditto.	Ditto.	Ditto.	

A 2 per cent., 1 per cent., or ½ per cent. dilution of "fixed rabies virus" in 0·5 per cent. carbolic acid normal saline solution is not destroyed in 24 hours at a temperature of 37°C; but a 4 per cent. dilution in 1 per cent. carbolic acid is destroyed in 24 hours at a temperature of 37°C.

TABLE VI.

In the series of six experiments recorded in this table the rabicidal properties of 1 per cent. carbolic acid at a temperature of 37°C were tested on 8 per cent. dilutions of rabies virus, with the result that in every instance the virus was killed in 24 hours. A vaccine prepared by this method and then diluted with an equal volume of sterile normal saline solution becomes 4 per cent. rabies virus in 0·5 per cent. carbolic acid normal saline solution; a very suitable strength of virus for antirabic treatment, containing sufficient carbolic acid to prevent any chance of subsequent contamination when stored for use after being made up.

The safety of such a vaccine is beyond dispute; so the next point to decide was, is it efficient in conferring immunity against the subdural inoculation of lethal doses of living rabies virus in animals, such as monkeys, dogs, and rabbits? The immunising experiments recorded further on furnish the answer to this question.

TABLE VI.

Experiments to test the effect of carbolic acid on "fixed rabies virus" when kept at a temperature of 37°C for 24 hours.

No. of experiment.	Percentage of "fixed virus" in normal saline solution used.	Percentage of carbolic acid added.	Time and temperature at which kept before being used.	Animal inoculated.	Amount inoculated, and method of inoculation.	Result.	Remarks.
	Per cent.	Per cent.					
1	8	1	24 hours at 37°C.	Rabbit	0·25 c.c., subdurally.	Remained well.	
2	8	1	Ditto.	Ditto.	Ditto.	Ditto.	
3	8	1	Ditto.	Ditto.	Ditto.	Ditto.	
4	8	1	Ditto.	Ditto.	Ditto.	Ditto.	
5	8	1	Ditto.	Ditto.	Ditto.	Ditto.	
6	8	1	Ditto.	Ditto.	Ditto.	Ditto.	

8 per cent. dilutions of "fixed rabies virus" in normal saline solution are killed in 1 per cent. carbolic acid in 24 hours at a temperature of 37° C.

CHAPTER IV.

The Effect of Normal Saline Solution on Rabies Virus.

Before proceeding to demonstrate the immunising properties of a 4 per cent. dilution of dead rabies virus in 0·5 per cent. carbolic acid, it is necessary to prove the effect of normal saline solution on rabies virus at room temperature, and also at a temperature of 37°C as some of the tests of immunity (such as the rabicidal properties of the serum) applied to the animals treated with dead rabies virus depend upon this point, *viz.*, whether dilutions of rabies virus are or are not quickly killed in normal saline solution.

The experiments of Lamb and McKendrick[1] on the effect of incubation at 37°C on 1 in 200, and 1 in 1,600 dilutions of rabies virus in normal saline soultion for various periods up to 24 hours show that the virus is partially destroyed after from 2 to 4 hours, and completely destroyed after 24 hours. My own experiments on this point failed to confirm those of Lamb and McKendrick (see Table IX).

1.—At Room Temperature.

TABLE VII.

The experiments in this table were carried out with a 1 per cent. dilution of "fixed virus" in normal saline solution, when kept at room temperature (about 20°C) for various periods up to 15 days.

All the animals inoculated subdurally with 0·2 c.c. of the virus kept under these conditions for 1, 2, 3, 4, and 11 days developed rabies on the 7th day, proving that no diminution of virulence had taken place up to that time. One animal was inoculated subdurally with 0·2 c.c. of the virus after it had been kept for 15 days, and rabies developed on the 9th day.

These results prove that rabies virus retains its virulence unimpaired in 1 per cent. dilutions in normal saline solution at room temperature for 11 days at least, and is not destroyed in similar dilutions in 15 days.

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[1] Lamb and McKendrick—Observations on Rabies. Scientific Memoirs by Officers of the Medical and Sanitary Departments of the Government of India, No. 36, pp. 27-32.

TABLE VII.

Experiments to test the effect of normal saline solution on "fixed rabies virus" when kept at room temperature (about 25°C).

No. of experiment.	Percentage of "fixed virus" in normal saline solution used.	Time and temperature at which kept before being used.	Animal inoculated.	Amount inoculated, and method of inoculation.	Result.	Remarks.
	Per cent.					
1	1	24 hours, room temperature, about 20° C.	Rabbit .	0·2 c.c. subdurally.	Rabies, 7th day.	
2	1	48 hours, room temperature, about 20° C.	Ditto.	Ditto.	Ditto.	
3	1	3 days, room temperature, about 20° C.	Ditto.	Ditto.	Ditto.	
4	1	4 days, room temperature, about 20° C.	Ditto.	Ditto.	Ditto.	
5	1	11 days, room temperature, about 20° C.	Ditto.	Ditto.	Ditto.	
6	1	15 days, room temperature, about 20° C.	Ditto.	Ditto.	Rabies, 9th day.	

A 1 per cent. dilution of "fixed virus" in normal saline solution is not destroyed in 15 days when kept at room temperature (about 20° C).

2.—At a Temperature of 37°C.

TABLE VIII.

The object of this series of experiments was to test the effects of 1 per cent. and 0·5 per cent. dilutions of rabies virus in normal saline solution when kept for 24 hours at a temperature of 37°C, then for 24 hours at room temperature, and finally for 24 hours in 0·5 per cent. carbolic acid at room temperature. The results show that a 1 per cent. dilution of virus when subjected to this treatment produced rabies in a rabbit on the 7th day after subdural inoculation with 0·3 c.c.; and that a 0·5 per cent. dilution of virus after similar treatment produced rabies in rabbits on the 8th day after subdural inoculation with 0·3 c.c.

A control rabbit inoculated subdurally with 0·3 c.c. of 1 in 200 virus kept for 24 hours at 37°C only, developed rabies on the 7th day.

It is evident from these results that a 1 in 200 dilution of rabies virus in normal saline solution is not destroyed in 24 hours at a temperature of 37°C; and that it can still further survive the effects of 0·5 per cent. carbolic acid for another 24 hours at room temperature.

TABLE VIII.

Experiments to test the effect of normal saline solution on "fixed rabies virus" when kept for 24 hours at 37°C, followed by 0·5 per cent. carbolic acid at room temperature for another 24 hours.

No. of experiment.	Dilution of "fixed virus" in normal saline solution.	Treatment to which it was subjected before being used.	Animal inoculated.	Amount inoculated, and method of inoculation.	Result.	Remarks.
1	1 in 100	24 hours at 37°C, then 24 hours at room temperature, and finally 24 hours in ½ per cent. carbolic acid at room temperature.	Rabbit .	0·3 c.c. subdurally.	Rabies, 7th day.	
2	1 in 200	Ditto.	Ditto.	Ditto.	Rabies, 8th day.	
3	Ditto.	Ditto.	Ditto.	Ditto.	Ditto.	
4 Control.	Ditto.	24 hours at 37°C .	Ditto.	Ditto.	Rabies, 7th day.	

1 per cent. and ½ per cent. dilutions of "fixed rabies virus" in normal saline solution are not destroyed when kept for 24 hours at a temperature of 37°C, then for 24 hours at room temperature, followed by another 24 hours in 0·5 per cent. carbolic acid at room temperature.

TABLE IX.

The results recorded in Table VIII clearly indicate that a temperature of 37°C. for 24 hours has no effect in diminishing the virulence of either a 1 per cent. or a 0·5 per cent. dilution of rabies virus in normal saline solution.

In order to leave no possible doubt on this point it was decided to carry out similar experiments, but with higher dilutions kept for various periods up to 24 hours at a temperature of 37°C. Two dilutions in normal saline solution were prepared from the medulla of a rabbit a few hours after the animal had died from fixed virus—one dilution 1 in 200, and the other 1 in 1,600—and both were placed in the incubator at a temperature of 37°C. Two control rabbits were inoculated subdurally with these dilutions immediately they were made up; one with 0·2 c.c. of the 1 in 200 dilution, and the other with 0·3 c.c. of the 1 in 1,600 dilution; and both animals developed rabies on the 7th day.

A series of rabbits were inoculated subdurally with 0·3 c.c. from the 1 in 200 dilution after being kept for 1, 2, 4, and 24 hours in the incubator at 37°C, and a

similar series with 0·3 c.c. from the 1 in 1,600 dilution also kept for 1, 2, 4, and 24 hours in the incubator at 37°C. The rabbits inoculated with the 1 in 200 virus kept for 1, 2, 4, and 24 hours at 37°C all developed rabies on the 7th day; and those inoculated with the 1 in 1,600 virus kept for 1, 2, and 4 hours also developed rabies on the 7th day.

Two rabbits inoculated with the 1 in 1,600 virus kept for 24 hours at 37°C developed rabies, one on the 10th day, and the other on the 11th day.

The results of this series of experiments prove that dilutions of 1 in 200 rabies virus in normal saline solution are not destroyed in 24 hours at a temperature of 37°C; and that dilution of 1 in 1,600 loses nothing of its virulence when kept for 4 hours at a temperature of 37°C, but has lost a little of its virulence although not destroyed at the end of 24 hours.

TABLE IX.

Experiments to test the effect of normal saline solution on "fixed rabies virus" when kept at a temperature of 37°C for various periods up to 24 hours.

No. of experiment.	Percentage of "fixed virus" in normal saline solution.	Time and temperature at which kept before being used.	Animal inoculated.	Amount inoculated, and method of inoculation.	Result.	Remarks.
1	1 in 200	Used when made up	Rabbit .	0·2 c.c. subdurally.	Rabies, 7th day.	Controls of experiments 3, 4, 5 and 6.
2	Ditto.	Ditto.	Ditto.	0·3 c.c. subdurally.	Ditto.	
3	Ditto.	1 hour at 37°C .	Ditto.	Ditto.	Ditto.	
4	Ditto.	2 hours at 37°C .	Ditto.	Ditto.	Ditto.	
5	Ditto.	4 hours at 37°C .	Ditto.	Ditto.	Ditto.	
6	Ditto.	24 hours at 37°C .	Ditto.	Ditto.	Ditto.	
7	1 in 1,600	Used when made up	Ditto.	Ditto.	Ditto.	Control of experiments 8, 9, 10, 11 and 12.
8	Ditto.	1 hour at 37°C .	Ditto.	Ditto.	Ditto.	
9	Ditto.	2 hours at 37°C	Ditto.	Ditto.	Ditto.	
10	Ditto.	4 hours at 37°C .	Ditto.	Ditto.	Ditto.	
11	Ditto.	24 hours at 37°C .	Ditto.	Ditto.	Rabies, 11th day.	
12	Ditto.	Ditto.	Ditto.	Ditto.	Rabies, 10th day.	

A 1 in 200 and a 1 in 1,600 dilution of "fixed rabies virus" in normal saline solution are not destroyed when kept at a temperature of 37°C for 24 hours.

CHAPTER V.

The Immunisation of Animals with Rabies Virus killed by Carbolic Acid, and their Subsequent Resistance to the Subdural Inoculation of Lethal Doses of "Street Virus," and also of "Fixed Virus."

The vaccines used in these immunising experiments were prepared from the brain and medulla of rabbits dead from "fixed virus" rabies. The dilutions of living virus used to test for immunity were prepared from the medulla. In no case was a vaccine or a test virus prepared from the spinal cord, as the cord contains less virus than the brain and medulla, and besides the virus in the cord may not be so evenly distributed as it is in the brain and medulla.

Nitsch[1] has shown that in "fixed virus" rabies the brain and medulla are more virulent (*i.e.*, contain more virus) than the spinal cord, and this has been confirmed by other workers.

In three of the four series of experiments here recorded, the virus was killed by exposing 8 per cent. dilutions in 1 per cent. carbolic acid normal saline solution to a temperature of 37°C for 24 hours. An equal volume of sterile normal saline solution was then added, and the mixture put aside until it was required for use. This gave a 4 per cent. dead virus in 0·5 per cent. carbolic acid.

In one series of experiments (Table IV, 4th series) on rabbits, the virus used was a 2 per cent. dilution in 0·5 per cent. carbolic acid, and killed in the same way as described above, *viz.*, by exposing a 4 per cent. dilution of fixed virus in 1 per cent. carbolic acid normal saline solution to a temperature of 37°C for 24 hours and then diluting with an equal volume of sterile normal saline solution.

The animals subjected to treatment were monkeys, dogs, and rabbits. In all cases the vaccine was injected hypodermically on the sides of the abdomen, and as far as possible a fresh site was selected each time. One dose of vaccine was given daily,—a small dose to begin with, and gradually increased as treatment advanced. During the treatment the animals remained in good health, and as they were well fed and attended to they increased in weight. In no case were any local or general effects noticed. Ordinary aseptic precautions were observed in administering the daily doses of vaccine.

The length of time which elapsed after the vaccine was prepared and before it was used varied from ten days to three weeks, and in no case was the vaccine used until it had been proved dead by the result of the subdural inoculation of a sample into a rabbit. As none of the rabbits which had been inoculated subdurally with 8 per cent. virus kept for 24 hours in 1 per cent. carbolic acid at a temperature of 37°C developed rabies, it may be accepted as proved that virus

[1] Nitsch—Wiener klinische Wochenschrift, No. 28 of 1904.

which has been subjected to this treatment is invariably killed. This opinion is based on the results of some 20 experiments.

The test of immunity applied to the animals after treatment consisted in the subdural inoculation of living virulent rabies virus ; and previous to this the serum was tested for its rabicidal properties. The results of these experiments are given in the tables which follow.

It is necessary to explain that the subdural inoculation of living rabies virus is a very severe test of immunity, and that it requires a very high degree of immunity to withstand this test. In the practical application of antirabic treatment, it is not necessary to confer such a high degree of immunity in order to prevent infection from the bites of rabid animals. I am sure that very few (if any) patients after undergoing an ordinary course of antirabic treatment at a Pasteur Institute would survive the subdural inoculation of living rabies virus, although they had acquired sufficient immunity to prevent the onset of hydrophobia after being bitten by rabid animals.

The fact that it is possible to immunise even a small percentage of animals treated with a rabies vaccine against the subsequent subdural inoculation of a living rabies virus is sufficient proof that such a vaccine is efficient.

It is a well known fact that no matter what method we adopt in immunising animals with rabies virus, only a percentage of them will be able to withstand the subdural inoculation of living virus. In the immunising experiments recorded in this paper, 8 animals were treated with dead virus, and afterwards trephined and inoculated subdurally with living virus, with the result that 6 remained well, and 2 developed rabies. The 2 which developed rabies, developed it at a later period than the control animals, proving that they were more resistant than the controls.

It is worthy of note that all the monkeys (four) treated with dead vaccine survived the subdural inoculation of living virulent virus. This result in itself is sufficient proof that the vaccine used was efficient, and there can be no manner of doubt about its safety.

The other test of immunity applied to the 8 animals treated with a dead rabies vaccine was the rabicidal properties of the serum.

Samples of blood were taken (in some cases once, and in other cases twice) before the animals were trephined for subdural inoculation, and when the serum had separated it was mixed with an equal volume of diluted living rabies virus, and the mixture placed in the incubator at a temperature of 37°C for 2 hours. After remaining for 2 hours at a temperature of 37°C, the mixture was removed, remixed, and 0·2 c.c. inoculated subdurally into rabbits.

In every case a control rabbit was inoculated with a similar preparation, except that the serum of a normal untreated animal of the same species as the immunised animal was used.

Altogether 12 experiments and 12 control experiments with serum were carried out as described above, with the result that evidence of complete rabicidal power was present in 7, and in the remaining 5 there was evidence of considerable rabicidal power, as the animals developed rabies at a later period than the control animals in which normal serum was used. The virus dilutions used in these rabicidal experiments were passed through a fine wire strainer to keep back any coarse particles of fibrous tissue which might be present, and in no case was the virus passed through a filter of any kind.

1st Series of Immunising Experiments.

In this series of experiments, two healthy full grown monkeys each weighing about 15 lbs. were immunised. They were the species of common brown monkey found in the hills in the neighbourhood of Kasauli, and had been in captivity for three or four months before being experimented on. Each received one inoculation daily for 24 days of a 4 per cent. dilution of dead rabies virus in 0·5 per cent. carbolic acid normal saline solution. The smallest dose given was 1 c.c. and the largest 3 c.c., and the increase from 1 to 3 c.c. was gradual. The total amount of vaccine injected was 50 c.c.

Both animals remained in good health during treatment and gained a little in weight.

Eleven days after completion of treatment, samples of blood were taken, and the serum tested for its rabicidal power on "fixed virus." One monkey's serum showed a complete rabicidal effect on an equal volume of 1 in 300 dilution of "fixed virus," when the mixture of serum and virus was kept for 2 hours at a temperature of 37°C, and the other monkey's serum an incomplete but marked rabicidal effect. (See experiments 1 and 2 in 5th series.)

Fifteen days after completion of treatment both monkeys were trephined and inoculated subdurally with 0·2 c.c. of a 1 in 500 dilution of 2nd passage "street virus," fresh from a rabbit's medulla a few hours after death from 2nd passage "street virus."

Both monkeys remained well.

Two control rabbits were inoculated subdurally with the same virus; one with 0·15 c.c. of 1 in 500 dilution, and the other 0·15 c.c. of 1 in 1,000 dilution. The former developed rabies on the 11th day, and the latter on the 12th day.

Another control rabbit inoculated subdurally with 0·2 c.c. of the virus used to immunise these monkeys remained well.

The results of this series of experiments prove that monkeys can be highly immunised against rabies by the hypodermic inoculation of dead rabies virus, when the virus has been killed and preserved in carbolic acid.

TABLE I.

1st Series of Immunising Experiments.

Experiments on monkeys to test the immunising properties of dead rabies virus. The virus was killed by exposing an 8 per cent. dilution of "fixed virus" in 1 per cent. carbolic acid normal saline solution to a temperature of 37°C for 24 hours; it was then diluted with an equal volume of normal saline solution, which gave 4 per cent. virus in 0·5 per cent. carbolic acid.

No. of experiment.	Duration of treatment.	Total quantity of 4 per cent. dead rabies virus injected subcutaneously.	Test to which subjected.	Result.	Remarks.
1. Monkey .	One injection daily for 24 days.	50 c.c. .	15 days after completion of treatment, was trephined and inoculated subdurally with 0·2 c.c. of 1 in 500 dilution of 2nd passage "street virus" fresh from a rabbit's medulla.	Remained well.	
2. Monkey .	Ditto.	Ditto.	Ditto.	Ditto.	
3. Rabbit. Control of test virus.	..	..	Trephined and inoculated subdurally with 0·15 c.c. of 1 in 500 dilution of the same virus used to test the two monkeys.	Rabies, 11th day.	
4. Rabbit, 2nd control of test virus.	..	..	Same as No. 3 experiment, with the exception that 1 in 1,000 dilution of test virus was used.	Rabies, 12th day.	
5. Rabbit. Control of immunising virus.	..	..	Trephined and inoculated subdurally with 0·2 c.c. of the virus used for immunising Nos. 1 and 2 monkeys.	Remained well.	

2nd Series of Immunising Experiments.

In this series two healthy full grown monkeys of a similar species to the two used for the 1st series of experiments were immunised. Each weighed about 16 lbs.; and they had been in captivity for three months before being experimented on.

Both received the same treatment, *viz.*, one hypodermic injection daily for 24 days of a 4 per cent. dilution of dead rabies virus in 0·5 per cent. carbolic acid normal saline solution.

The smallest dose given was 1 c.c. and the largest 3½ c.c., and the increase from 1 c.c. at the outset to 3½ c.c. later on was gradual. No change was apparent in the condition of their health during treatment.

Fourteen days after the completion of treatment samples of blood were taken to test for rabicidal properties of the serum. The test applied to the serum of these two monkeys was carried out in a manner similar to the test applied to the serum of the two monkeys referred to in the 1st series of experiments, except that a 1 in 200 dilution of 1st passage human virus, fresh from the medulla of a rabbit which had died from subdural inoculation of a portion of the brain of a hydro-

TABLE III.

3rd Series of Immunising Experiments.

Experiments on dogs to test the immunising properties of dead rabies virus. The virus was killed by exposing an 8 per cent. dilution of "fixed virus" in 1 per cent. carbolic acid normal saline solution to a temperature of 37°C. for 24 hours; it was then diluted with an equal volume of normal saline solution, which gave 4 per cent. virus in 0·5 per cent. carbolic acid.

No. of experiment.	Duration of treatment.	Total quantity of 4 per cent. dead rabies virus injected subcutaneously.	Test to which subjected.	Result.	Remarks.
1. Dog, weight 24 lbs., full grown.	One injection daily for 28 days.	100 c.c.	24 days after completion of treatment, was trephined and inoculated subdurally with 0·3 c.c. of 1 in 600 dilution 2nd passage jackal's virus, fresh from a rabbit's medulla.	Remained well.	
2. Puppy dog, weight 13 lbs.	Ditto.	90 c.c.	Ditto.	Rabies, 29th day.	
3. Control dog, weight 14 lbs. full grown.	..	..	Trephined and inoculated subdurally with 0·3 c.c. of 1 in 600 dilution of same virus used to test Nos. 1 and 2 dogs.	Rabies, 10th day.	
4. Rabbit. Control of immunising virus.	..	..	Trephined and inoculated subdurally with 0·2 c.c. of the virus used for immunising Nos. 1 and 2 dogs.	Remained well.	

4th Series of Immunising Experiments.

The experiments in this series were carried out on rabbits. Two healthy full grown rabbits each weighing 2½ kilogrammes were immunised with 2 per cent. dilutions of dead rabies virus in 0·5 per cent. carbolic acid normal saline solution. They received one hypodermic injection daily for 24 days. The doses varied from 0·5 c.c. at the outset, to 2 c.c. later on, and the increase from the smaller to the larger doses was gradual. The total amount of vaccine given to each animal was 36 c.c.

On the 8th day after completion of treatment the serum of both animals gave a complete rabicidal effect when mixed with a 1 in 200 dilution of "fixed virus" and kept for 2 hours at a temperature of 37°C.

On the 25th day the serum was again tested on a 1 in 200 dilution of "fixed virus" when the rabicidal effect was found to be complete in one, and incomplete, but marked in the other.

On the 25th day after completion of treatment both rabbits were trephined and inoculated subdurally with 0·25 c.c. of a 1 in 200 dilution of 2nd passage "street

virus '' fresh from the medulla of a rabbit dead from 2nd passage '' street virus '' rabies. One rabbit remained well and the other (the one whose serum gave a incomplete rabicidal effect on the 25th day) developed rabies on the 16th day.

A control rabbit inoculated subdurally with a 1 in 400 dilution (*i.e.*, half the strength) of the same virus used to test the two immunised animals developed rabies on the 10th day. Another control rabbit inoculated subdurally with 0·25 c.c. of this virus used to immunise the two rabbits remained well.

The results of this series of experiments show that rabbits can be immunised with hypodermic injections of dead rabies virus ; and of two rabbits immunised by this method one attained a degree of immunity sufficient to withstand the subdural inoculation of virulent rabies virus, and the other almost succeeded in attaining this degree of immunity.

The test applied to these two rabbits was a very severe one, *viz.*, the subdural inoculation of 0·25 c.c. of a 1 in 200 dilution of virus. A more highly diluted virus such as 1 in 600 or 1 in 1,000 would have been a fairer test.

TABLE IV.

4th Series of Immunising Experiments.

Experiments on rabbits to test the immunising properties of dead rabies virus. The virus used was killed by exposing a 4 per cent. dilution of " fixed virus " in 1 per cent. carbolic acid normal saline solution to a temperature of 37°C. for 24 hours ; it was then diluted with an equal volume of normal saline solution, which gave 2 per cent. virus in 0·5 per cent. carbolic acid.

No. of experiment.	Duration of treatment.	Total quantity of 2 per cent. dead rabies virus injected subcutaneously.	Test to which subjected.	Results.	Remarks.
1	One injection daily for 24 days.	36 c.c.	25 days after completion of treatment was trephined and inoculated subdurally with 0·25 c.c. of 1 in 200 dilution of 2nd passage " street virus " fresh from a rabbit's medulla.	Remained well.	The test applied to these two experiments (Nos. 1 and 2) was a severe one, as the virus used was diluted only to 1 in 200.
2	Ditto	Ditto	Ditto ditto	Rabies, 16th day.	
3. Control of test virus.	..	..	Trephined and inoculated subdurally with 0·25 c.c. of 1 in 400 dilution of same virus used for experiments 1 and 2.	Rabies, 10th day.	
4. Control of immunising virus.	..	..	Trephined and inoculated subdurally with 0·25 c.c. of virus used for the immunising experiments 1 and 2.	Remained well.	

CHAPTER VI.

The Evidence of Immunity in the Serum of Animals treated with Rabies Virus killed by Carbolic Acid.

SERIES V.

EXPERIMENTS TO TEST THE RABICIDAL PROPERTIES OF THE SERUM OF ANIMALS IMMUNISED WITH DEAD RABIES VIRUS.

This series of experiments gives the rabicidal properties of the serum of the eight animals (four monkeys, two dogs, and two rabbits) immunised with 4 per cent. dead virus in 0·5 per cent. carbolic acid normal saline solution referred to in series I, II, III, and IV.

In each of these experiments blood was taken in glass capsules and put aside at room temperature for 24 hours until the serum had separated. The serum was then pipetted off and mixed with an equal volume of 1 in 200 or 1 in 300 dilution of living rabies virus in normal saline solution as recorded opposite each experiment. The mixture was then placed in an incubator at a temperature of 37°C for two hours, after which it was re-mixed and 0·2 c.c. inoculated subdurally into rabbits. In each case a control rabbit was inoculated subdurally with 0·2 c.c. of an exactly similar mixture as regards the dilution of virus and kind of virus used, except that the serum added was from a normal monkey, dog, or rabbit, as the case might be and kept for the same time (2 hours) at 37°C.

On referring to the table (Series V) it will be seen that 12 experiments were carried out with the serum from 8 immunised animals, and that in 7 of these experiments the serum showed a complete rabicidal action, and in 5 an incomplete but marked action.

It was a mistake perhaps, not to have filtered the rabies emulsions before adding them to the serum to be tested. They were only passed through a fine wire strainer to keep back coarse particles of fibrous tissue. However, the results as they stand show a complete rabicidal action in 7, and a well marked action in 5.

In the 12 control experiments the animals developed rabies after an incubation period which corresponded to the normal incubation period of the virus used.

The results of these experiments prove that the serum of animals immunised with rabies virus killed and preserved in carbolic acid has a well marked rabicidal effect on living rabies virus.

When we examine the results recorded in Tables VII, VIII and IX, in which the effects of normal saline solution on various dilutions of rabies virus at a temperature of 37°C and at room temperature are given, it is evident that the normal saline solution used to dilute the virus was not the cause of the rabicidal action attributed to the serum in Series V.

In 1891 Babes demonstrated the fact that the serum of animals immunised against rabies had a well marked rabicidal action on living rabies virus *in vitro*.

Marie[1] mentions that Kraus and Kreissl have studied the rabicidal properties of the serum of patients during and after completion of Pasteur's method of antirabic treatment and found evidence of rabicidal properties 22 days after the completion of treatment, but not during or immediately after treatment.

Lamb and McKendrick,[2] in 1908, tested the serum of patients whom they had treated by Högyes' dilution method of treatment, and failed to obtain any evidence of rabicidal properties in the serum.

In 1903-04-05, when Director of the Pasteur Institute of India, I carried out a series of experiments on the serum of patients after undergoing Pasteur's method of treatment, and obtained evidence of well marked rabicidal effects a few days after completion of treatment. In another series of experiments carried out at the same time in which two ponies were highly immunised by commencing with dead virus, and going on to living "fixed virus," the serum was so highly rabicidal that one volume added to two volumes of a 5 per cent. dilution of fixed virus completely destroyed its virulence in ten minutes at room temperature.[3]

Babes, Remlinger, Marie and other workers have obtained similar evidence of the rabicidal properties of the serum of animals immunised with rabies virus.

The serum of normal untreated man or animals does not furnish any evidence of rabicidal action.

It is evident from these experiments that the rabicidal action of the serum of animals treated with a rabies vaccine is one of the factors which indicate immunity.

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[1] Marie—L'Etude Experimentale de la Rage 1909, page 172.

[2] Lamb and McKendrick—Scientific Memoirs of Officers of the Medical and Sanitary Departments of the Government of India, No. 36, pp. 12—15.

[3] Semple—On the Preparation and Use of Antirabic Serum, and on the Rabicidal Properties of the Serum of Patients after undergoing antirabic treatment.—*Lancet*, June 6th, 1908, p. 1611.

TABLE V

RABICIDAL PROPERTIES OF SERUM

Experiments on rabbits to test the rabicidal properties of the serum of animals immunised with dead rabies virus

No. of experimental animal	Injected intracerebrally	Interval between injection of virus and injection of serum	Injected intracerebrally	Result	Remarks
1 Monkey	1 per cent virus in 0·5 per cent carbolic acid	11 days	1 equal part serum and 1 in 100 fixed virus kept 5 hours at 37° C.	Rabies 10th day	Control rabbit inoculated intracerebrally with 0·5 c.c. equal parts normal monkey serum and 1 in 100 fixed virus kept 5 hours at 37° C. developed rabies on the *10th day*
2 Monkey	Ditto	11 days	Ditto	Ditto	Rabies 10th day	Ditto
3 Monkey	Ditto	14 days	1 equal part serum and 1 in 100 fixed virus kept 5 hours at 37° C.	Ditto	Rabies 17th day	A control rabbit inoculated intracerebrally with 0·5 c.c. equal parts normal monkey serum and 1 in 100 fixed virus kept 5 hours at 37° C. developed rabies on the *10th day*
4 Monkey	Ditto	14 days	Ditto	Ditto	Remained well	Ditto	Ditto
5 Dog	Ditto	11 days	1 equal parts serum and 1 in 300 fixed virus kept 5 hours at 37°C.	Ditto	Ditto	A control rabbit inoculated intracerebrally with 0·5 c.c. equal parts normal dog serum and 1 in 100 fixed virus kept 5 hours at 37° C. developed rabies on the *9th day*
6		13 days	1 equal parts serum and 1 in 100 emulsion of fixed virus mixed and kept 5 hours at 37° C.	Ditto	Rabies 11th day	A control rabbit inoculated intracerebrally with 0·5 c.c. equal parts normal dog serum and 1 in 300 and passage rabbit virus kept 5 hours at 37° C. developed rabies on the *11th day*
7 Dog	Ditto	11 days	1 equal part serum and 1 in 300 fixed virus mixed and kept 5 hours at 37° C.	Ditto	Remained well	A control rabbit inoculated intracerebrally with 0·5 c.c. equal parts normal dog serum and 1 in 300 fixed virus kept for 5 hours at 37° C. developed rabies on the *7th day*
		13 days	1 equal part serum and 1 in 300 passage rabbit virus mixed and kept 5 hours at 37° C.	Ditto	Rabies 11th day	A control rabbit inoculated intracerebrally with 0·5 c.c. equal parts normal dogs serum and 1 in 300 and passage rabbit virus kept 5 hours at 37°C. developed rabies on the *11th day*
7 Rabbit	2 per cent virus in 0·5 per cent carbolic acid	8 days	1 equal parts serum and 1 in 100 fixed virus kept 5 hours at 37°C.	Ditto	Remained well	A control rabbit inoculated intracerebrally with 0·5 c.c. equal parts normal rabbits serum and 1 in 100 fixed virus kept 2 hours at 37° C. developed rabies on the *7th day*
		25 days	Ditto	ditto	Ditto	Ditto	Ditto	ditto
8 Rabbit	Ditto	8 days	Ditto	ditto	Ditto	Ditto	Ditto	ditto.
		25 days	Ditto	ditto	Ditto	Rabies, 10th day	Ditto	Ditto

All these experiments with the serum from immunised rabbits dogs and monkeys were controlled with serum from normal rabbits, dogs and monkeys and treated exactly the same after being mixed with the same dilutions of virus as the serum which was being tested. All the control rabbits contracted rabies after the usual incubation period according to the virus used.

CHAPTER VII.

The advantages of having a safe and efficient Antirabic Vaccine which could be sent to wherever it is required for the treatment of patients.

Antirabic treatment is essentially a prophylactic treatment, but it differs from the prophylactic treatment applied to other infectious diseases in that it is carried out only when the patient has already been infected, and before the infection has had time to assume an active phase. On this account it is most important when treatment is necessary, that it should be commenced as early as possible.

A person infected with rabies virus cannot afford to allow the virus to get a good start in its growth and then leisurely to make his way to a Pasteur Institute for treatment.

In a country like India where rabies is so widely spread, and the distances from a Pasteur Institute are great, a certain amount of delay is unavoidable. Those who are slightly bitten on the limbs, or through clothing, can better afford delay than those who are severely bitten, or bitten on the head, neck or face

The fact that a large percentage of persons bitten by rabid animals escape hydrophobia even without treatment, indicates that it does not require a very high degree of immunity to prevent the virus from multiplying and giving rise to hydrophobia. Then again, the fact that equally good results are claimed for any and every method of antirabic treatment, also indicates that a very high degree of immunity is not an absolute necessity in prophylactic treatment.

To my mind the two essential factors in any prophylactic vaccine are :—

1. That it should be an absolutely safe vaccine.
2. That it should be an efficient vaccine, *i.e.*, capable of giving rise to an immunising response sufficient to prevent the onset of the disease for which it is given

The first of these factors deserves careful consideration when we are dealing with a living vaccine, and especially a living rabies vaccine, where infection with the living element in the vaccine would mean certain death to the patient.

As I have already stated, Pasteur, when he first applied antirabic treatment to man, got over this difficulty by means of his "dried cord" method of treatment. His well known method of obtaining "fixed virus" from the spinal cords of rabbits only requires to be mentioned here. Suffice it to say that after a variable number of passages of "street virus" (a virus of variable virulence) through a series of rabbits, a virus of a constant and exalted virulence is invariably present in the spinal cord (and also in the brain and medulla) of these animals. When the cords are removed after death from "fixed virus" and dried over caustic potash

in glass jars, kept in a dark room at a temperature of 22°C the virus has lost all its virulence in 14 days ; or in other words, it is then a dead virus. Cords which have been dried for a lesser period than 14 days give very little evidence of virulence after the 8th or 9th day, and have not lost anything in virulence until after the 3rd day. There are two opinions as to what happens to the virus in the cords during this drying process. One opinion is, that the virus becomes attenuated, and strictly in accordance with the time the cord has been dried ; and the other is, that the virus is only diminished in amount and not attenuated.

It is a well known fact that germs which can be exalted in virulence can also be attenuated in virulence, and that drying is one method of attenuation. It is also accepted as a fact that rabies is caused by a germ of some kind, and a germ which can be exalted in virulence ; why then should rabies virus be the exception to a rule which is applicable to the causal organisms of other infectious diseases in regard to its attenuation ?

As the subject is one about which there are sure to be differences of opinion until it becomes possible to cultivate the causal organism of rabies in artificial media we had better leave it with this explanation ; moreover, the acceptance of one or other of these opinions does not materially concern the subject-matter of this paper.

Pasteur commenced treatment by injecting vaccines prepared from cords which had been dried for 14 days, and then from cords which had been dried for 13, 12, 11, 10 days, and so on down the scale to cords which had been dried for 3 days only. The dose for an adult consists of 1 c.m. of cord finely ground down in 3 c.c. of a sterile fluid, such as normal saline solution, and injected subcutaneously ; it corresponds to about a 5 per cent. cord emulsion.[1] This method modified in various directions is what is known as the " dried cord " method of treatment. It has undergone many modifications since Pasteur's time, but the system is still essentially that of Pasteur.

In the " dilution method " of treatment (also known as Högyes' method) the vaccines are prepared direct from the fresh cord of a rabbit dead from " fixed virus " rabies.

It is convenient to begin by making a 1 per cent. dilution of virus, and from this to prepare any dilution required. The treatment by this method is commenced by the injection of very weak dilutions, such as 1 in 6,000 at some Institutes, and 1 in 4,000, or even 1 in 2,000 in others ; and gradually going on from day to day to a less diluted virus, such as a 1 in 500 ; 1 in 200 ; or even 1 per cent. dilutions at some Institutes.

[1] Harvey and McKendrick—Scientific Memoirs by the Officers of the Medical and Sanitary Departments of the Government of India, No. 30, p. 19.

It will thus be seen that in the " dried cord " method, treatment is commenced by a dead virus, and finally a virulent virus is used. In the " dilution method " treatment is commenced by a very small quantity of a living virulent virus, and increasing its amount as treatment advances.

Equally good results are claimed for both methods and their modifications; but with these results I am not concerned in the present paper which deals solely with the safety and efficiency of a dead virus. The fact that living vaccine is used in both methods would preclude the possibility of sending it to a distance for the treatment of patients without running serious risks of interfering with its efficiency; whereas a dead carbolised vaccine could well be sent to any centre where treatment could be carried out without running any risks of injuring its properties. The experiments recorded in this paper in which a dead carbolised rabies virus was used to immunise monkeys, dogs and rabbits, prove that it is a vaccine which can be relied upon to produce a high degree of immunity; and knowing that it is a dead vaccine we can dismiss any doubts as to the possibility of its producing the disease which it is intended to prevent. It is also less expensive than any other method, and is easily carried out.

A vaccine prepared from the brain and medulla of " fixed virus " rabbits would contain more of the active element (virus) than a vaccine prepared from the spinal cords, and on this account its immunising properties would be greater than cord vaccine, and moreover the brain and medulla of a rabbit would furnish a much larger amount of vaccine than the spinal cord.

One Central Pasteur Institute in addition to carrying out treatment could supply the whole of India with a vaccine ready to be administered to patients at other centres. By this means patients would be saved long and expensive journeys; and most important of all, they would come under early treatment, and a treatment free from risks.

CHAPTER VIII.

Summary of Conclusions.

1. Rabies virus is easily killed by heat. A temperature of 50°C is sufficient to kill a 5 per cent. dilution of "fixed virus" in 15 minutes.

2. Rabies virus can remain alive and virulent for several days in normal saline solution.

A 1 per cent. dilution of "fixed virus" in normal saline solution remains alive and virulent for at least 15 days at room temperature (about 20°C). Dilutions of 1 in 200, and 1 in 1,600 of "fixed virus" in normal saline are not killed in 24 hours at a temperature of 37°C, and not even injured in any way in 4 hours at this temperature. Dilutions of 1 per cent. rabies virus in normal saline solution are not killed when kept for 24 hours at a temperature of 37°C, then for 24 hours at room temperature, and finally for 24 hours in 0·5 per cent carbolic acid at room temperature.

3. Rabies virus can survive for a longer time in carbolic acid at room temperature (about 20°C) than at a temperature of 37°C. It is also more resistant to carbolic acid at both these temperatures than most non-spore forming bacteria.

4. A one per cent. dilution of rabies virus in 0·5 per cent. carbolic acid normal saline solution remains alive and virulent for at least 20 days at room temperature (about 20°C). One per cent. and 2 per cent. dilutions of rabies virus in 1 per cent. carbolic acid normal saline solution remain alive and virulent for at least 4 days at room temperature (about 20°C).

5. Dilutions of 4 per cent. and 8 per cent. rabies virus in 1 per cent. carbolic acid normal saline solution are killed in 24 hours at a temperature of 37°C. Rabies virus killed by this method and then diluted with an equal volume of sterile normal saline solution is a safe and efficient antirabic vaccine.

6. Animals such as monkeys, dogs, and rabbits can be highly immunised by hypodermic injections of a rabies vaccine killed and preserved in carbolic acid. Of 8 animals, *viz.*, 4 monkeys, 2 dogs, and 2 rabbits, immunised with a 4 per cent dead virus in 0·5 per cent. carbolic acid, all the monkeys, one dog, and one rabbit survived the subdural inoculation of lethal doses of virulent living virus; and the one dog and one rabbit which did not survive this test showed a longer incubation period than the controls.

7. The serum of animals immunised with rabies vaccine killed and preserved in carbolic acid gives a well marked rabicidal action on living virulent virus.

8. A rabies vaccine prepared from the brain and medulla of "fixed virus" rabbits, and then killed and preserved in carbolic acid is a safe and efficient vaccine in the immunisation of animals, as proved by experiments on monkeys, dogs, and rabbits; and judging from the results of these experiments it should also prove a safe and efficient antirabic vaccine for the prophylactic treatment of persons bitten by rabid animals.

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